Biomarkers to Assess Possible Biological Effects on Reproductive Potential, Immune Function, and Energetic Fitness of Bottlenose Dolphins Exposed to Sounds Consistent with Naval Sonars

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LONG-TERM GOALS

The overarching goal of project N000141110432 is to utilize novel biomarkers to examine whether significant sublethal responses to sonar-type sounds occur in bottlenose dolphins exposed to such sounds. The collaborators will use immune function markers, acute phase proteins, fertility potential assays, and targeted and non-targeted metabolomics to investigate samples collected from trained dolphins before exposure to the sonar-type sounds, immediately after exposure, and one week post-exposure.

OBJECTIVES

Science is an iterative process. The same questions tend to be re-examined as tools of greater sophistication and higher resolution are used. Whereas marine mammal responses to stress have often historically been examined using rather blunt and indirect tools including some behavioral responses, today's molecular technologies, in the form of biomarkers, provide avenues by which scientists can directly measure biologically significant responses such as reproductive potential, immune system function, acute phase responses, and energetic fitness. The tools we use include a range of biomarkers of effects, many of which have been developed and calibrated for human medical uses, but which work well in other mammals. The key is to design collaborative teams that (a) acquire appropriate samples for analysis, (b) conduct R&D to ensure that available assays can measure useful parameters, (c) design experiments in which animals are humanely exposed to stressors and an extensive suite of biological effects is monitored, (d) conduct chemical assays that rigorously adhere to QA/QC requirements; and (e) interpret the results of assays with regard to what they do or do not mean in terms of potential biologically significant effects on populations.

APPROACH

Sampling protocols: In fall 2009, U.S. Navy scientists (SSC Pacific) exposed 19 Navy dolphins maintained in San Diego, California, to sounds that are consistent with those produced by mid-frequency active (MFA) sonar. The signals (~3.5kHz) produced received levels ranging from 115 to 185 dB re 1 μPa. Subjects received up to 10 exposures within a 5 minute period, depending on their

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willingness to participate. All appropriate permits and IACUCs were in place prior to the exposure testing.

A cross-over experimental design was used for the exposure studies, such that individual dolphins served as their own controls, thereby increasing the power of the analyses for the discovery of new predictive biomarkers of stress. This entailed collection of a series of serum samples from individuals subjected to acoustic stresses.

For 18 dolphins used in the tests, there are pre-test samples, samples taken immediately following the exposure session, and samples taken approximately one week after the exposure. For one of the subjects, only the samples taken immediately following and one week following the exposure exist.

Clinical Chemistry: We will follow our established protocols for using clinical chemistry to assess liver and kidney function (as well as other traditional, proven parameters of animal health) as a framework against which to assess the novel biomarkers being used in this study. Dr. Andrew Stamper will interpret the health data. Assays typically done (through a contract with Sarasota General Hospital) include: Complete Metabolic Panel (NA, K, CL, CO2, glucose, BUN, creatinine, calcium, ALK.phos, AST(SGOT), ALT(SGPT), total bilirubin, total protein and albumin); iron, LDH; phosphate; and uric acid. For liver function assays AST, ALT, total bilirubin, and uric acid are most relevant, whereas for kidney function, BUN and creatinine are of particular interest. For general inflammation markers, we will use sedimentation rate and fibrinogen levels.

Immune function and acute phase markers: For assays of immune function and acute phase markers, prepared kits will be acquired from Bio-Rad. Immune function will be assessed using a 27-cytokine kit, and acute phase responses will be evaluated using a 4-plex kit.

The assays will be done using a Luminex Bio-Plex 3D. This instrument combines existing technology in flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry to assess up to 500 different analytes simultaneously.

Inasmuch as we are using prepared kits for which protocols have been established for other species (human and non-human), and since the different kits follow slightly different protocols, our assays will follow the manufacturers instructions.

Metabolomic biomarkers: Non-targeted metabolomics will be conducted using direct infusion Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS). Previously we have developed and utilised this approach for measuring a diverse range of metabolites in several different sample types. Here, metabolites within the previously frozen serum samples will be extracted using a methanol:chloroform:water solvent system. The polar metabolite fraction will then be aliquoted, dried, and stored at -80 degrees C until analysis. On the day of mass spectrometric analysis, samples will be resuspended in methanol:water 4:1 with 0.25% formic acid (for analysis in the positive ion mode) and in methanol:water 4:1 with 10 mM ammonium acetate (for the negative ion mode). FT-ICR mass spectrometry will be performed using a LTQ FT Ultra instrument (Thermo Fisher Scientific, Bremen, Germany) equipped with a chip-based direct infusion nanoelectrospray ion source (Triversa, Advion Biosciences, Ithaca, NY). Spectra will be acquired in triplicate (per sample) from a 96-well plate using the SIM stitching method, from *m/z* 70 to 590 in both ion modes. The resulting spectra will be processed using in-house software and analyzed using multivariate statistics (e.g., principal

components analysis, partial least squares discriminant analysis) in order to discover metabolic biomarkers that change concentration in response to sound stress.

Targeted metabolite analysis will be conducted using a highly sensitive, specific and quantitative approach implemented on an LC triple quadrupole mass spectrometer. These targeted profiling studies will focus on the measurement of steroids and steroid-like molecules (including stress hormones such as cortisol) that are known to play a role in the stress responses of marine mammals. Mass spectrometry is well accepted as being a reliable, quantitative tool for the analysis of steroids; as evidence for this statement, a Web of Science search of research publications revealed that 2,621 papers have been published on this topic during the past years (using "steroid" and (mass spectrometry or LC-MS or GC-MS or LC/MS or GC/MS)" for the keyword search). Here we will optimise our extraction protocols using solid phase extraction (e.g. using Waters OASIS HLB cartridges), and then implement LC-MS/MS analyses on our Thermo Fisher Scientific TSQ Vantage triple quadrupole mass spectrometer. This will involve optimizing the ionization conditions (we will use nanoelectrospray ionization to enhance sensitivity over traditional approaches); selecting appropriate multiple reaction monitoring (MRM) transitions, as well as optimizing the internal standards used for metabolite quantification (we will use deuterium labeled steroids as internal standards, for which we have previous experience).

Fertility potential assays: In the assays being conducted for AMH and inhibin-B, standards, controls, and serum samples will be incubated in microtitration wells coated with the appropriate antibody. After incubation and washing, the wells will be treated with another detection antibody labeled with biotin. After a second incubation and washing step, the wells will be incubated with streptavidin-horseradish peroxidase (HRP). After a third incubation and washing step, the wells will be incubated with the substrate tetramethylbenzadine (TMB). An acidic stopping solution will then be added and the degree of enzymatic turnover of the substrate determined on a plate reader by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured is directly proportional to the concentration of hormone present. A set of AMH or inhibin-B standards will used to plot standard curves of absorbance vs. hormone concentration, and samples will be run in duplicate. Based on the standard curves, the concentration of AMH or inhibin-B in the dolphin serum samples will be calculated.

The ELISA assays will be run using an automated DSX Automated Plate Processor. This instrument is capable of handling up to four plates (96 samples/plate) and performing multiple different assays on each plate.

WORK COMPLETED

Activities and accomplishments in 2011 (March-September 2011):

Permitting and sample disbursement: The samples we will use for the study have been archived through collaborator, Dr. Dorian Houser. Samples will ultimately be sent to both Mote Marine Laboratory for assays of immune function, fertility potential, and clinical chemistry (volumes permitting) and to University of Birmingham for targeted and non-targeted metabolomics.

- The samples destined for Mote Marine Laboratory have been sent by Dr. Houser and received by the PIs. Analyses are being initiated (see below).

- Sending samples out of the United States (i.e., to the UK and University of Birmingham) requires appropriate CITES permits. Co-PI Reynolds has spoken with Dr. Robert Brownell (NMFS) who has an institutional permit that will permit the easy, but completely legal transfer of samples to colleagues in the UK. This will eliminate the time-consuming need for the project PIs to acquire a separate CITES permit. The samples have not been sent as of the submission of this report, but Drs. Houser and Brownell will likely be able to send the samples to Viant during the month of September 2011.

Analyses: One of Dr. Viant's doctoral students from University of Birmingham is working in Dr. Wetzel's laboratory at Mote Marine Laboratory for the next 4+ months. She has received special training on the Bio-Plex 3D system and is currently in the process of starting to run the samples received from Dr. Houser; her focus is a multiplexed cytokine assays which provide more than two dozen measures of immune function. The PIs have agreed that she can use the data as a component of her doctoral degree. Although some results are available, they have not been interpreted to date.

- Dr. Viant is initiating a search for a postdoctoral scientist who specializes in metabolomics to conduct the targeted and non-targeted assays in the laboratory at University of Birmingham.

Orientation: As noted, one of Dr. Viant's students has received special training to work with Dr. Wetzel on multiplexed immune function assays of the dolphin serum samples provided by Dr. Houser.

- In addition, Drs. Houser, Wetzel and Reynolds all participated in a workshop held to bring together recipients of ONR funding to assess effects of stress on marine mammals. The "ONR Marine Mammal & Biology Program, ONR Marine Mammal Stress Program Kick-Off Meeting" was held in Arlington, Virginia on 20 July 2011 and provided an effective venue for exchange of ideas and creation of useful new ties among professionals with related interests.

Critical next stapes for 2012: Next steps include sending complete samples to Dr. Viant in the UK, hiring of a metabolomics post-doctoral scientist at University of Birmingham, conducting the immune function assays, the fertility potential assays and clinical chemistry analyses at Mote Marine Laboratory.

RESULTS

The samples sent to Mote Marine Laboratory just recently arrived; the samples destined for University of Birmingham have going to be shipped imminently. Even the Mote Marine Laboratory samples have not been run at this point, so there are no data or results to report.

IMPACT/APPLICATIONS

The conduct of this study will provide a number of basic and applied benefits with regard to science and management, as follows: provide development of new tools for understanding the biology of bottlenose dolphins and other marine mammals and provide a clarification of the real extent of sublethal harm.